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Summary of Unfunded Continuation of Project:

The work on human neuromas, uncovering cellular and molecular events in neuroma formation and developing models of human neuroma, has proceeded with great productivity over the past year.

Project 1: The first project developed a new way of thinking about human neuromas. In this study, we combined biological parameters with patient parameters (manuscript submitted.) That is, we compared the levels of axonally transported proteins into nerves of the neurons, and important patient parameters such as pain, the type of injury, location of injury and other outcome variables after surgery. For the first time, this project has been able to associate biological variables such as the level of Gap-43 and P-75, two important transported proteins that are associated with the response of peripheral nerves to injury with pain. In this study, we were able to carry out quantitative western blot analysis for the proteins Gap-43 and P-75. (Gap-43 is made in the dorsal root ganglion and P-75 is made in the dorsal root ganglion and spinal motor neuron.) It was found that there is a high correlation between the amount of interaxonal Gap-43 and the post-injury time interval, the time after injury to surgery. This was a highly significant correlation $p=0.0038$. In addition, there was a significant relationship between high levels of Gap-43, the type of injury, transection or stretch, and the sensation of pain by the patient. This approach may be one of the most fruitful in that it is able to combine outcome variables and examine neurovariables at the time of surgery. Moreover, in the future, it may provide predictors for the long term outcome. A larger study and a grant application are anticipated for further work.

Project 2: The purpose of this study was to develop a model for avulsion injury of the rat brachial plexus (manuscript submitted.) Brachial plexus injuries are among the most incapacitating and the likelihood of successful repair is small. These injuries particularly affect people around machinery, heavy equipment, automobile accidents and infants. At this time, there are no good models of animal injuries of this type as the existing animal models have required invasive procedures and they are unspecified in their degree of damage. We have developed a new model using passive acceleration which very closely mimics the type of mechanical event that occurs to the human injury. This model is highly predictable so that we are able to specify with a high degree of accuracy the number of roots that are being avulsed and also the level at which they are being avulsed. This model should be extremely valuable to develop behavioral outcomes with therapeutic interventions and also to more closely examine the molecular events that are involved in the loss of function after this type of injury.

Project 3: This project was an examination of the expression in the spinal cord of an immediate early gene, c-Fos, after brachial plexus injury and peripheral nerve injury. This manuscript has been published in "Neurosurgery" (attached) this past year and it is the first effort to compare the effects of injury at different levels of the interaction with the peripheral nerve and the spinal cord. The manuscript showed, in a quantitative fashion, the importance of the time and the site in the spinal cord where injury was noted by the up regulation of c-Fos using the quantification of neurons and cells expressing c-Fos proteins. The value to this approach is that it began to examine in a rigorous fashion how neurons would begin to respond at early times with injuries at different levels in the peripheral nerve spinal cord system.

These projects have developed two very important themes, biological and patient variables in neuroma injury and the non-invasive spinal cord injury model, that will be followed up with additional work and it is expected that these will lead to separate applications for additional funding from foundations such as the National Institutes of Health.

Expression of c-Fos Protein in the Spinal Cord after Brachial Plexus Injury: Comparison of Root Avulsion and Distal Nerve Transection

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OBJECTIVE: Clinical reports indicate poor outcomes for avulsion injuries, compared with more peripheral nerve damage. These two different injuries may both affect gene expression in spinal neurons, and the changes in gene expression may be related to the types of injuries.

METHODS: The brachial plexus of 48 adult male rats was lesioned by either root avulsion close to the spinal cord or distal nerve transection. The rats were quickly revived and remained awake until death at 30, 60, or 120 min after surgery. In rats with avulsive injuries, traumatic sites on the dorsal and ventral horns of the spinal cord were microscopically detected. Immunocytochemical analysis of the c-*fos* product was performed for the two experimental groups and for sham-treated control animals at the same survival times.

RESULTS: An increase in Fos-like immunoreactivity (FLI) in cells of the spinal cord, at levels C4–T1, was detected at 30 min after nerve transection or root avulsion. The number of FLI-positive cells continued to increase at 60 and 120 min after the nerve injury ($P = 0.001$). FLI-positive cells were compared at the C7 level, in laminae 1 and 2, 3 and 4, and 5 to 10, after the two injuries and were found to be more abundant after the avulsive injury ($P = 0.0001$); furthermore, the number of FLI-positive cells increased with time ($P = 0.001$). In a comparison of all levels, both experimental groups demonstrated significantly greater numbers of FLI-positive cells than did controls, and the group with nerve root avulsion showed significantly ($P = 0.0001$) more FLI-positive cells than did the group with distal nerve transection.

CONCLUSION: These results suggest that nerve root avulsion from the spinal cord leads to increased and prolonged expression of c-*fos* and, potentially, greatly increased transcription of new messages for recovery, survival, or cell death. (Neurosurgery 42:1357–1363, 1998)

Key words: Brachial plexus injury, *c-fos*, Immediate-early gene, Nerve root avulsion, Nerve transection, Spinal cord injury

Peripheral nerve damage is often part of the situation resulting from severe extremity injuries. Avulsion of one or more roots from the spinal cord occurs in approximately 70% of severe brachial plexus stretch avulsion injuries (42). Nerve section at a distance from the dorsal root ganglion and spinal cord has classically been found to result in chromatolysis of sensory and motor neurons. Molecular responses in dorsal root ganglion cells result in increased transcription of novel messenger ribonucleic acids for the production of structural and membrane proteins that regulate the consequences of and recovery from injury (11, 12). In contrast, severe injuries to the proximal brachial plexus in the neck or shoulder or the lumbosacral plexus in the pelvis might have more deleterious long-term consequences. Permanent but variable loss of motor or sensory functions are common findings among such patients. Retrograde damage to neurons resulting from injuries close to the plexus can often be serious enough to be incompatible with neuronal survival (16).

Traumatic or ischemic injuries to the central nervous system initiate reactive molecular and biochemical changes, some of which are autodestructive and others that are neuroprotective (41). After peripheral nerve injury or various types of stimulation, an immediate-early gene, *c-fos*, appears rapidly within cells of the spinal cord (1, 3, 4, 8, 10, 18, 21, 25, 36, 40).

This gene codes for the Fos protein products, which form the Fos/Jun dimer, and binds to the consensus AP-1 (activator protein 1) binding site, regulating the expression of other genes (26). Previous studies have shown that transection or crushing of the rat sciatic nerve induces expression of c-*fos* in the dorsal root ganglia, spinal cord, and higher centers (3, 5, 6, 9). However, the expression of c-*fos* has not been investigated after injuries close to the spinal cord, such as brachial plexus avulsive injuries, which result in very serious and complex neurosurgical problems and poor outcomes (20).

Variable time courses for the increases in c-Fos protein levels in the spinal cord under different pathological conditions have been described (30). The largest change in Fos-like immunoreactivity (FLI) was found to be within 2 hours after peripheral nerve injury or noxious stimulation (6, 32). In the present study, the brachial plexus nerves of rats were injured either by avulsive injury of the brachial plexus close to the spinal cord or by distal transection of the branches of the brachial plexus. The expression of Fos protein in the rat spinal cord was investigated by using an immunocytochemical method, and the number of neurons revealing FLI were quantified to compare the differences in neuronal responses in the spinal cord after these two types of injury.

MATERIALS AND METHODS

Animal model and surgical procedure

Forty-eight male Sprague-Dawley rats (270–350 g) were anesthetized by halothane inhalation. The brachial plexus (C4–T1) was exposed from the outlet of the vertebrae down to the axilla, by a ventral midline incision extending to the axilla on both sides. After the skin and subcutaneous tissues had been cut, the sternohyoideus muscles were separated from the midline. The muscles were retracted laterally so that the cervical vertebrae could be exposed. The fourth to eighth cervical nerves and the first thoracic nerve were carefully separated from the paraspinal muscles. Small nerve branches, such as the dorsalis scapulae, suprascapularis, and subscapularis nerves, were cut as far distally as possible. Other nerves, such as the musculocutaneous, axillary, thoracicus, medianus, ulnaris, and radialis nerves, were separated and prepared for use. The following procedures were applied to rats randomly assigned to one of two groups. In the first group, a small hemostat was used to lightly hold the nerves of C4–T1 about 1 cm lateral to the spinal cord. The nerves were pulled away from the spinal cord within 1 second. To control the extent of injury, the dorsal root ganglia were mobilized in the process. This procedure was shown to stretch the roots away from the spinal cord with the expected avulsive injury. In the second treatment group, the nerves were cut, with sharp scissors, about 2 cm lateral to the spinal cord. In sham-treated control rats, incisions were made without muscle or nerve injury. The time for the operation was about 10 min. The rats began to move their lower limbs and to awaken about 3 minutes after discontinuation of halothane administration. There was no movement of the upper limbs after either avulsion or transection of the plexus, whereas sham-treated control animals moved their limbs well.

Specimen processing and immunocytochemistry

The rats were anesthetized, at 30, 60, or 120 minutes after injury or sham operation, with phenobarbital (administered intraperitoneally). The animals were then perfused through the heart with 250 ml of saline, followed by 250 ml of 4% paraformaldehyde. The spinal cord at levels C4–T1 was removed, postfixed in 4% paraformaldehyde for 4 hours, and immersed for 24 hours in 30% sucrose in phosphate-buffered saline. Before sectioning, each segment of the spinal cord from C4 to T1 was labeled with a number 5 suture, under an operating microscope. The junction between two segments was determined by distinguishing the outlets of the roots. Frozen sections (40 μm) were cut and processed for the avidin-biotin immunohistochemical procedure, by using a peroxidase Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and the protocol suggested by the manufacturer. For detection of FLI, the sections were incubated with a 1:500 concentration of a polyclonal antibody to Fos protein (PC05; Oncogene Science, Uniondale, NY). Control sections were incubated in the presence of Fos peptides (PC09 and PC10; Oncogene Science) to which the antibodies had been raised. After completion of the diaminobenzidine reaction, sections were air dried, mounted, and covered with coverslips.

Quantification of neurons with FLI

The sections were studied with dark-field and bright-field microscopy, and projection drawings were made using a camera lucida attachment (1, 29). To map the pattern of FLI-expressing cells, an outline of the C4–T1 segments was drawn and the distribution of FLI-positive neurons was plotted on the drawing under dark-field illumination. FLI-positive cells in C4–T1 spinal cord segments were counted. Labeled cells from three random histological sections in each segment were counted and averaged. Individual counts of grouped laminae were made to discern potential differences between the sensory and motor systems; laminae were grouped as follows: 1 and 2, 3 and 4, and 5 to 10. The counts were made by an investigator (YP) who was blinded to the particular treatment the animals had received. Statistical analysis of the data was performed by using the Statistical Analysis System program (SAS Language Reference, Version 6; SAS Institute, Cary, NC) on an IBM-compatible computer. Analysis of variance was performed to compare the effects of the sham procedure and the two treatments (nerve section and nerve avulsion), with respect to the segment level of the spinal cord and also the time after injury. The level of significance considered to be statistically significant was at $P < 0.05$. Protected t tests of least-squares means derived from the analysis of variance were used to compare the interactions among treatment, spinal cord level, and time, as well as the differences between the laminae. The means and standard errors were processed with Excel (Microsoft, Seattle, WA), to plot the histograms.

RESULTS

Spinal cord injury

At the time of animal death, the spinal cord and dorsal root ganglia were examined with the dura intact; it was observed

that the sham and distal nerve transection procedures had no effect on the normal structural appearance, as seen under an operating microscope. On the other hand, after the avulsion procedure, subdural bleeding was seen in every rat. Examination of cross-sections of the spinal cord at C4–T1 in rats after avulsive injury revealed that both the dorsal and ventral horns were traumatically injured. Bleeding could also be seen within the spinal cord (*Fig. 1, C* and *D*). Most lesions on the dorsal horn were found in superficial laminae 1 and 2. There were many neurons located within 100 μm of the lesion sites. Lesion sites were also detected within the gray matter of the ventral horn (*Fig. 1D*).

Fos protein expression

In normal rats (n = 2) that had been quickly killed by pentobarbital administration, without any operative procedures, few FLI-positive cells were seen in spinal cord segments C4–T1. However, the baseline statistical comparisons were made with a group of sham-treated control animals that had undergone the same halothane anesthesia, skin incision, and revival for 30, 60, or 120 minutes.

Sham-treated control rats exhibited a slight but nonsignificant increase in the number of FLI-positive neurons in the medial half of the superficial dorsal horn (*Fig. 1*). These segments corresponded to the termination sites of cutaneous nerve afferents from the incision area (5, 39).

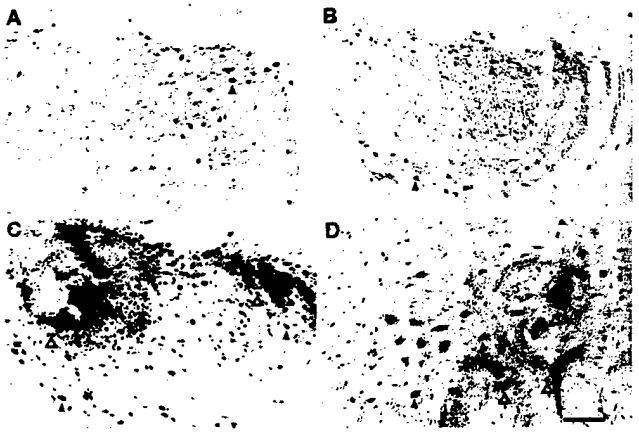

FIGURE 1. Photomicrographs from the C7 segment, showing examples of FLI-positive neurons from rats 2 hours after surgery. *A*, dorsal horn of a rat after a sham operation; FLI-positive neurons appeared in the medial half of the superficial spinal cord. *Arrowhead*, FLI-positive neuron. *B*, dorsal horn of a rat after distal transection; FLI-positive neurons (*arrowheads*) appeared mainly in the superficial laminae. *C*, dorsal horn of a rat after nerve root avulsion; there were many FLI-positive neurons in the superficial laminae near the lesion sites. *Closed arrowheads*, FLI-positive neurons; *open arrowheads*, lesion sites. *D*, ventral horn of a rat after root avulsion, with many FLI-positive motor neurons near the lesion sites. *Closed arrowheads*, FLI-positive neurons; *open arrowheads*, lesion sites. *Scale bar*, 32 μm.

Both experimental groups demonstrated greater FLI than did the sham-treated control animals. FLI detection in the spinal cord showed increased numbers of cells exhibiting c-Fos protein expression 30 min after surgery ($P = 0.0001$). These levels continued to rise at 60 and 120 minutes after the nerve injury (*Fig. 2*). The increased numbers of cells expressing FLI appeared mainly in spinal cord laminae 1 to 7 and 10, with the greatest number in laminae 1 and 2 (*Fig. 3*). This distribution was similar from 30 to 120 minutes after either nerve root avulsion or nerve transection. However, FLI increased more rapidly after nerve avulsion than after distal sectioning. Thirty minutes after the procedures, the number of positive neurons was significantly greater for animals with nerve avulsion than for those with nerve sectioning. Differences continued to exist at 60 and 120 minutes after surgery (*Figs. 2* and *3*). There were more FLI-positive neurons in superficial to deep laminae of the spinal cord in rats with nerve root avulsion, compared with distal transection, especially in laminae 1 and 2 ($P = 0.0001$) (*Fig. 4*).

In rats with nerve root avulsion, there were many neurons expressing Fos protein in laminae 1 and 2 close to the lesion site at spinal cord levels C6 and C7 of the dorsal horn (*Fig. 1*). Expression of c-*fos* in the motor neurons was less distinctly

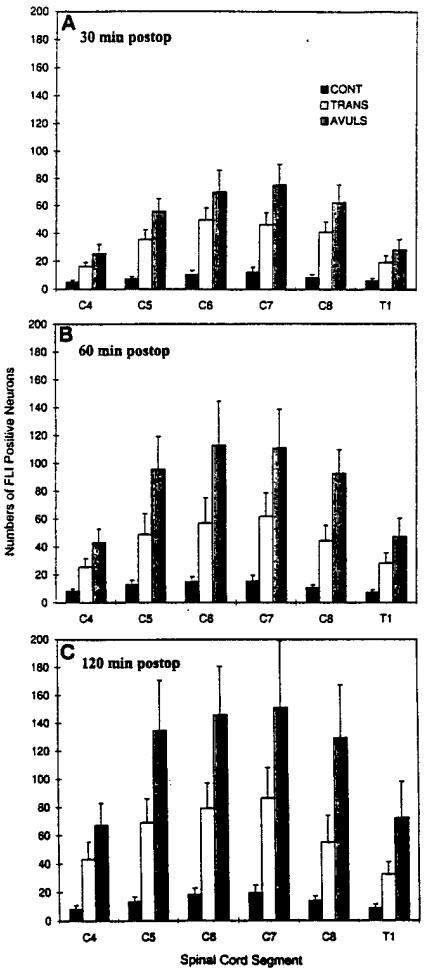

FIGURE 2. Histograms of the mean numbers of FLI-positive cells in response to sham operation (*CONT*) (n = 4), distal transection (*TRANS*) (n = 8), or nerve root avulsion (*AVULS*) (n = 8). *A*, 30 min after surgery; *B*, 60 min after surgery; *C*, 120 min after surgery. Means and standard errors are shown.

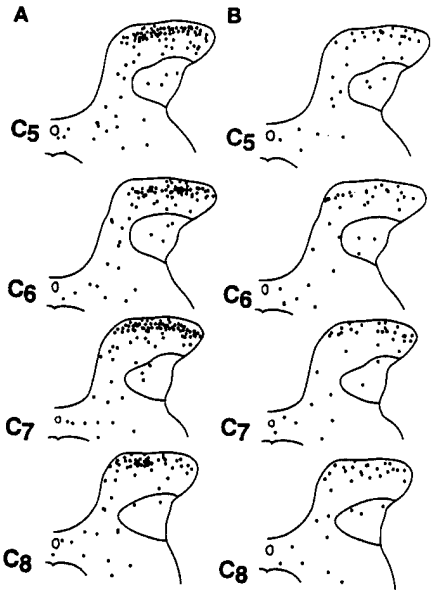

FIGURE 3. Camera lucida drawing, showing the FLI in the spinal cord at C5–C8. *Closed circles*, FLI-positive cells. *A*, avulsive injury; *B*, distal nerve transection. Note that there are more labeled cells in the superficial dorsal horn than in the deeper layers of the spinal gray matter. Moreover, there are more labeled cells in the animals with avulsive injuries (*A*) than in those with distal nerve transection (*B*).

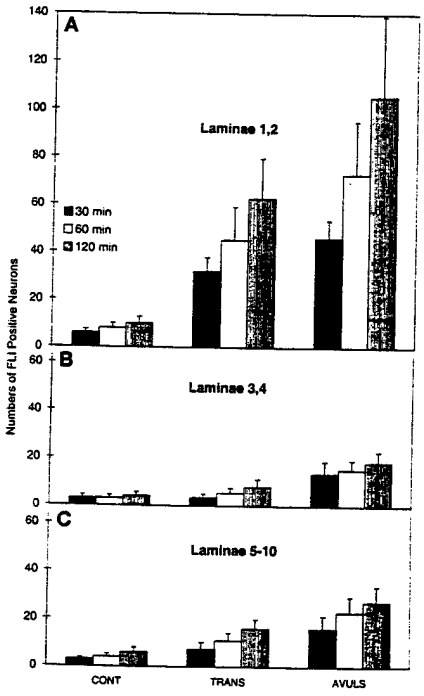

FIGURE 4. Histograms of the numbers of FLI-positive cells in different laminae of the spinal cord. *A*, numbers of FLI-positive neurons (mean ± standard error) in superficial laminae 1 and 2 at different times. *B*, numbers of FLI-positive neurons (mean ± standard error) in laminae 3 and 4 at different times. *C*, numbers of FLI-positive neurons (mean ± standard error) in laminae 5 to 10 at different times. *CONT*, sham-treated control; *TRANS*, distal transection; *AVULS*, nerve root avulsion.

detected in rats with distal nerve transection, but many motor neurons were found to express c-*fos* in rats that had undergone nerve root avulsion (*Fig. 1*).

Analysis of the FLI-positive cell counts after injury showed that, overall, the avulsion injury was significantly ($P = 0.0001$) more effective in stimulating c-*fos* expression in more cells at all times (means ± standard errors of cell numbers were 29.4 ± 0.3 and 19.5 ± 0.3 for avulsion and nerve sectioning, respectively). Overall, the number of cells expressing FLI was also significantly greater in the avulsive group when the effects of the two injuries were considered at the three times after injury ($P = 0.0001$). The primary effect on evoking FLI was seen at the C6 and C7 levels (*Figs. 1* and *2*). Moreover, at 2 hours, FLI expression was significant ($P = 0.0001$) for all of these comparisons, except for the 30-minute cell count at the C7 level, which was significant at $P = 0.0023$.

The increased numbers of cells expressing FLI appeared mainly in spinal cord laminae 1 to 7 and 10, with the greatest number in laminae 1 and 2 (*Figs. 1* and *4*). Analysis of the laminae at the C7 spinal cord level clearly showed the effects of mechanical disruption of the cells of the dorsal horn, compared with the more ventral neurons. Comparisons of laminae 1 and 2, 3 and 4, and 5 to 10 at the three times showed that the laminae and experimental injury were significant ($P = 0.01$) and that the increased expression from 30 to 120 minutes was significant at the $P = 0.0001$ level. However, detailed statistical analysis of the data showed that this was primarily because of increased FLI in laminae 1 and 2 at 60 and 120 minutes (comparisons with laminae 3 and 4 and 5–10 found these to be significant at the $P = 0.001$ level). Analysis of laminae 5 to 10 at all times showed no difference between avulsion and nerve transection at any of these times. In this study, the special motor neurons were counted as part of the neurons in laminae 7 to 9; the results were not statistically significant, because of the small number of motor neurons compared with the much larger number of sensory neurons. However, it was apparent by inspection of the slices that there was more FLI expression in motor neurons after the avulsive injury, compared with distal transection.

DISCUSSION

Brachial plexus injury is commonly found after trauma produced by acceleration of the head, neck, and upper limbs (20, 42). This injury usually causes a serious functional problem in the upper extremities of the patient. The current study provides evidence that avulsion close to the spinal cord might cause bleeding in the intradural cavity and spinal cord injury. FLI in the surviving neurons of the spinal cord is also a marker to map the changes in neuronal activity after noxious or nonnoxious stimulation (3, 4, 9, 26, 27, 32). An increase of c-*fos* expression has been demonstrated in the lumbosacral spinal cord of rats after sciatic nerve transection or crushing (5, 6, 19, 31). In this study, we detected elevated levels of Fos-like protein in the cervical spinal cord of rats after either nerve root avulsion or distal transection.

The possible roles of Fos production are many. It has been suggested that c-*fos* may participate in the regulation of increased opioid gene expression in the spinal cord (10). Activation of opioid-sensitive circuitry by electroacupuncture can suppress c-*fos* expression (21). Anesthetic agents and systemically administered morphine can suppress c-*fos* expression, and local anesthetic blockade of these neurons significantly decreased the number of FLI-positive neurons (5, 28, 32). In the current study, although the animals underwent nerve injury under anesthesia, they rapidly regained consciousness,

which decreased the effects of anesthesia on c-Fos protein expression studied after injury.

Local stimulation by electricity or *N*-methyl-D-aspartate can induce c-*fos* expression in spinal neurons (34). In the current study, in animals with distal transection of spinal nerves, only the primary sensory neurons and motor neurons were directly physically stimulated. However, in animals with nerve root avulsion, postsynaptic neurons located in the dorsal horn were also directly physically stimulated, because of the injury to the spinal cord. Therefore, c-*fos* expression in rats after nerve root avulsion was more intense than that after distal nerve transection.

After axonectomy, apoptosis might be initiated in lesioned neurons (2, 15, 22). Apoptosis is a highly regulated process that finally leads the neurons to death. Apoptosis can be identified by cell shrinkage, chromatin condensation, and cellular fragmentation (35). It is interesting that some proteins that function as modulators in cell proliferation and differentiation also regulate the apoptotic process (14, 33). The Fos protein appears rapidly within spinal neurons after nerve injury (1, 19). This protein has been shown to function as an activator to control subsequent transcription of other genes, the products of which could be required for long-term changes in neuronal excitability and repair (28, 38). More recently, c-Fos protein has been demonstrated to function also as a primary modulator of apoptotic cell death or programmed death (7, 33, 37). It was reported that transection of the sciatic nerve induced both increased expression of c-*fos* and evident apoptosis in the spinal neurons (17). Our study showed that, in the early stages of peripheral nerve injury, avulsion close to the spinal cord could damage the spinal cord gray matter, suggesting more intense physical stimulation of the spinal neurons, compared with distal nerve transection. This pronounced induced c-*fos* expression in the neurons of the spinal cord might play a role in the neuronal apoptotic process, which could lead more neurons to death in the nerve root avulsion model than in the distal nerve transection model. Such losses of cells in the gray matter of the spinal cord are especially responsible for permanent motor function deficits.

The relationship between neuronal degeneration and the remaining dendrite length after dendrite transection has been investigated in cell culture (13, 23, 24). Dendrites were amputated at lesion distances of either 50 or 100 μm from their perikarya. Neurons with dendritic transection showed ultrastructural damage, which spread from the transection site toward the perikaryon within 15 minutes. The probability of cell survival was closely related to the remaining dendrite length. Neurons with longer remaining dendrites showed higher survival rates. The current study showed that, in nerve root avulsion, the spinal cord can be seriously damaged and neurons in the dorsal and ventral horns can be injured. Many neurons might be axotimized within 100 μm of the cell bodies and might not survive. Massive expression of c-*fos* in these cells might indicate that the Fos protein plays a role in the neuronal degeneration and death seen with spinal cord injuries. Therefore, significantly higher c-*fos* expression after avulsive injury, compared with distal nerve transection, indicates direct effects of avulsive injury on spinal neurons, which may precede either cell recovery or cell death.

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COMMENTS

The authors present some interesting data regarding the increased expression of c-*fos* after root avulsion or distal nerve transection in a rat model. It is acknowledged that the types of lesions used in this report are difficult to standardize, particularly in attempts to produce the same avulsion injury each time. However, the data indicating variations in the expression of c-*fos* are clear and significant.

What is less clear is the potential difference in cells with Fos-like immunoreactivity (FLI). The introductory section quantifies FLI without regard to the types of cells involved, whereas the Discussion focuses on FLI in neurons. It is presumed that the majority of FLI detected in these specimens was localized to neurons; otherwise, the implications regarding apoptosis are somewhat limited.

The c-*fos* gene is implicated in a number of regulatory mechanisms within cells, and certainly it could be anticipated that trauma would be a potential basis for induction of immediate-early genes. The implications of these findings for therapy are limited, because the events described in this report would take place before any opportunity for intervention. Nevertheless, the authors have provided insights into the molecular events associated with root avulsion.

Joseph M. Piepmeier
New Haven, Connecticut

In this observational study, the authors demonstrated that FLI in neurons was increased after root avulsion or root transection at the cervical level in male Sprague-Dawley rats. Although this increase in FLI suggests that immediate-early genes are

turned on by these injuries and subsequent transcription of new genes might occur, such findings were not demonstrated in this study. Clearly, additional work is required to establish the functional significance of these findings.

Corey Raffel
Rochester, Minnesota

Zhao et al. have very clearly shown the effects of brachial plexus avulsion versus nerve transection on FLI in the rat spinal cord. They have shown that brachial plexus avulsion produces a much more profound effect than does more distal nerve transection. The effects were much greater in the dorsal and sensory neurons than in the ventral or motor neurons of the spinal cord. Because the c-Fos protein might play a role in apoptosis, the findings of Zhao et al. might help explain the poor recoveries seen after brachial plexus avulsion, compared with more distal nerve transection. It is hoped that, in the future, we will be able to manipulate these transcription pathways involved in cell death and to improve recovery via molecular mechanisms. Unfortunately, we still seem to be at the stage of characterizing the molecular responses, and our ability to manipulate these responses is limited.

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Toronto, Ontario, Canada

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A MODEL FOR AVULSION INJURY IN THE RAT BRACHIAL PLEXUS USING PASSIVE ACCELERATION.

R.J. Spinner, A. Khoobehi, S. Kazmi, S. Zhao, D.G. Kline*, Z. Zhang, R.W. Beuerman.
Dept of Neurosurgery, LSU Eye Center, Neuroscience Center of Excellence, LSU Medical Center School of Medicine in New Orleans; New Orleans, LA 70112.

We have developed an experimental model for brachial plexus injuries in the rat that closely simulates the characteristics of human injury and produces avulsion injuries in a non-invasive manner. A prototype apparatus was designed which allowed a force to be transmitted to a restrained limb with passive acceleration; this model facilitated a quantitative determination of the physical and mechanical parameters of the injury. The relationship between the test weight and the number of roots avulsed was found to be highly correlated and consistent over a body weight of 350 gm to 450 gm (correlation 0.92, $p<0.05$). Of the four roots affected (C5, C6, C7, and C8), C6 was avulsed in 29 animals (90.6%), C7 in 24 animals (75%), C8 in 9 animals (28.1%), and C5 in 5 animals (15.6%). The amount of force correlated to both the particular root(s) and the number of root(s) avulsed. This model of brachial plexus injury may be useful to further our understanding of the cellular response to this incapacitating injury and to develop therapeutic strategies with behavioral correlates EY04074 (NIH, NEI); DAMD17-93-V-3013 (Department of the Army, DOD).

Key Words: (see instructions p. 4)

1. Trauma
2. Dorsal Root
3. Dorsal horn
4. Spinal

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A Model for Avulsion Injury in the Rat Brachial Plexus Using Passive Acceleration

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Grant sponsor: Department of the Army Cooperative Agreement
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Grant #: EY04074 and EY02377 (Departmental Core Facility)

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Running title: A model for avulsion injury

Key words: avulsion injury, brachial plexus

A Model for Avulsion Injury in the Rat Brachial Plexus Using Passive Acceleration

Abstract

We have developed an experimental model for brachial plexus injuries in the rat that closely simulates the characteristics of human injury and produces avulsion injuries in a non-invasive manner. A prototype apparatus was designed which allowed a force to be transmitted to a restrained limb with passive acceleration. Reproducible results were obtained in the 32 rats that correlated the test weight and the number of roots avulsed ($r=0.92$; $p=0.0007$). The amount of force also correlated to the pattern of avulsion injury. A 230g weight produced either C6 (84.6%) or C7 (15.4%) avulsions; a 330g weight produced C6 (18.1%), C7 (9%) or C6 and C7 (72%) avulsions; a 530g weight produced C5 through C8 (75%) or C6 through T1 (25%) avulsions. This model of brachial plexus injury may be useful to further our understanding of the cellular response to this incapacitating injury and to develop therapeutic strategies with behavioral correlates.

Introduction

Currently, two open methods are used experimentally to produce a nerve root avulsion in the rat: an intravertebral (1-7) and an extravertebral method (8). Avulsion is produced by traction either directly on the exposed spinal root (1-7) or indirectly on a nerve exposed more distally (8). While both of these methods can produce injury at a known spinal cord level, they are done so in an invasive manner. These two methods are qualitative in nature in that the exact forces and the length of application are not well defined and exact reproductions of a particular injury are difficult. In addition, they do not reproduce the mechanisms proposed in brachial plexus avulsion which is a sudden, powerful force rapidly moving an upper limb forward. Alterations resulting from the surgery to produce the avulsion prevent accurate behavioral tests, neurological assessment and the development of new therapeutic strategies.

The goal of this project was to develop a closed method for producing an avulsive brachial plexus injury in the rat with predictable results. This method might then be used to develop therapeutic manipulations and behavioral correlates.

Materials and Methods

A prototype apparatus was designed using a passive acceleration method (PAM), which recreates a sharp ictus at the limit of the movement. This device simulates the mechanism which produces this type of injury in humans. Approval for use of animals in this experimental series is provided by the Louisiana State University Medical Center Institutional Animal Care and Use Committee.

Sprague-Dawley rats weighing between 300g and 500g were studied. They were anesthetized first with Halothane followed by an intramuscular injection of ketamine (90mg/kg) and xylazine (13 mg/kg). The rats were placed on their left side, within a custom designed 6 cm tube in diameter (Fig 1). The left forelimb protruded from a cutout on the undersurface of the tube. In this position, the limb made approximately a 180 degree angle with the front and back of the torso. The elbow was placed in a custom built two-part metal sleeve and was tightly secured to a 36 cm lever arm. The attachment was made so that the site where the force was applied (at 32 cm) was four times the distance from the fulcrum as the sleeve (at 8 cm). The forelimb supported the weight of the 35cm rod. A force was applied to the limb by dropping a predetermined test weight down the guide rod from a height (30cm) onto the lever. The apparatus was constructed to accommodate animals up to about 500g. Special care was taken so that the sleeve fit tightly and would not slip following the impact. Several design features addressed variability in rat weight and forelimb size. These modifications included sleeve inserts and an adjustable lever height.

Thirty-six rats were randomized into 4 groups using test weights of 230g, 330g, and 530g and a sham group. Overall, the weights tested in this group of 32 animals were as follows: 13 animals (40.6%) at 230 gms, 11 animals (34.4%) at 333 gms and 8 animals (25%) at 530 gms.

Shams (n=4) were placed in the apparatus and their left forelimbs were attached to the lever for 1 minute without dropping a weight.

All of the animals were sacrificed while under the initial anesthesia. The specimens were numbered and examined in a blinded manner to determine the pattern of brachial plexus injury. A laminectomy was performed and the spinal cord and the spinal nerves bilaterally were exposed from the occiput to the upper thoracic region using the operating microscope. The condition of the spinal cord and the spinal nerves bilaterally was noted. Correlation values and statistical significance were then determined using Stastistica (Stastistica release 5, StatSoft, Inc, Tulsa, OK 74104).

Results

The relationship between the test weight and the number of roots and the pattern of roots avulsed was found to be highly correlated and consistent over a body weight of 309 to 490 gms (mean = 363g +/- standard error = 9g). As shown in Table 1, the reliability of this model was shown by the correlation (r=0.92; p=0.0007) between the test weight and the number of roots avulsed. In this study, one root was avulsed in 12 animals (37.5%), two roots in 12 animals (37.5%), and four roots in 8 animals (25%). C5 was avulsed in 5 animals (15.6%), C6 in 29 animals (90.6%), C7 in 24 animals (75%), C8 in 9 animals (28.1%), and T1 in 2 animals (6.5%). The 230 g test weight produced injuries only of C6 (84.6%) or C7 (15.4%); 330g produced C6 (18.1%), C7 (9%) or C6 and C7 (72%) avulsions; 530g produced four root avulsions in all cases: either C5 throughC8 (75%) or C6 through T1 (25%). All of the avulsed nerves had that occur preganglionically at the level of the rootlets, and this involved ventral as well as dorsal roots. Except for three cases where the avulsion was partial, ventral and dorsal roots were avulsed

completely. Extensive hemorrhage was found at the avulsion sites on the spinal cord. The amount of blood tended to correlate with the force of impact. The sham group did not have any avulsions. In 4 of 8 cases where impact energy was highest, fractures of the radii and ulnae occurred. Fractures did not occur at lower energy levels.

Discussion

The data from these series of experiments demonstrate a reproducible pattern of root avulsions. There was a statistically significant correlation between the impact energy, the affected root and the number of spinal roots avulsed. These results also demonstrate the relative susceptibility of the C6 and C7 nerve roots to avulsion in our model, with relative protection of the C5 and T1 nerve roots (except at the highest impact); this corresponds to the frequent C7 injury seen in humans with longitudinal traction to the arm in abduction (9,10). Furthermore, the site of avulsion at the level of the rootlets also corresponds to the weakest link of the plexus in humans (10).

PAM has important advantages over other models in that it utilizes a definite impact-like force. This type of force is similar to the one described for human brachial plexus palsies from birth trauma or from motorcycle or automobile accidents (11). It allows for a quantitative rather than a qualitative approach to experimental brachial plexus injuries (12) in the rat. Similar to open models, this closed model can produce a predictable pattern of injury. In some preliminary studies, using a test weight of 530g, we have determined the acceleration to be approximately $5 m/sec^2$ and the force to be 0.6 joules.

Not all of the energy of the impact is transferred to the forelimb because of frictional forces on the rod (when the impact weight slides down) and at the fulcrum of the lever.

Furthermore, not all of the energy transferred to the forelimb goes into stretching the brachial plexus nerves. Energy is dissipated due to the fit of the sleeve around the forelimb in different animals and energy is absorbed by other anatomic structures in the forelimb. Nevertheless, the data show that among rats of similar weights, the variability with this experimental method is small and evidently constant.

Conclusion

The results of this study show that the passive acceleration method is a valid model for inducing avulsive brachial plexus injuries in rats. Using this method, ventral and dorsal spinal root avulsions can be done in a quantified, non-invasive, and reproducible manner.

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Legends

Figure 1 - 2-dimensional schematic, 3-dimensional rendered view of the custom made apparatus designed for this experiment. The anesthetized rat is placed within the tube (B). The forelimb protrudes from a hole in the bottom of the tube. The forelimb is held by a sleeve device that is connected to the lever arm (A). Doughnut-shaped weights are dropped down a circular rod (C).

Table 1 – The correlation ($r=0.92$) between the number of avulsed roots and the test weights can be seen. Each dot represents more than 1 animal and there are 32 data points plotted on this graph.

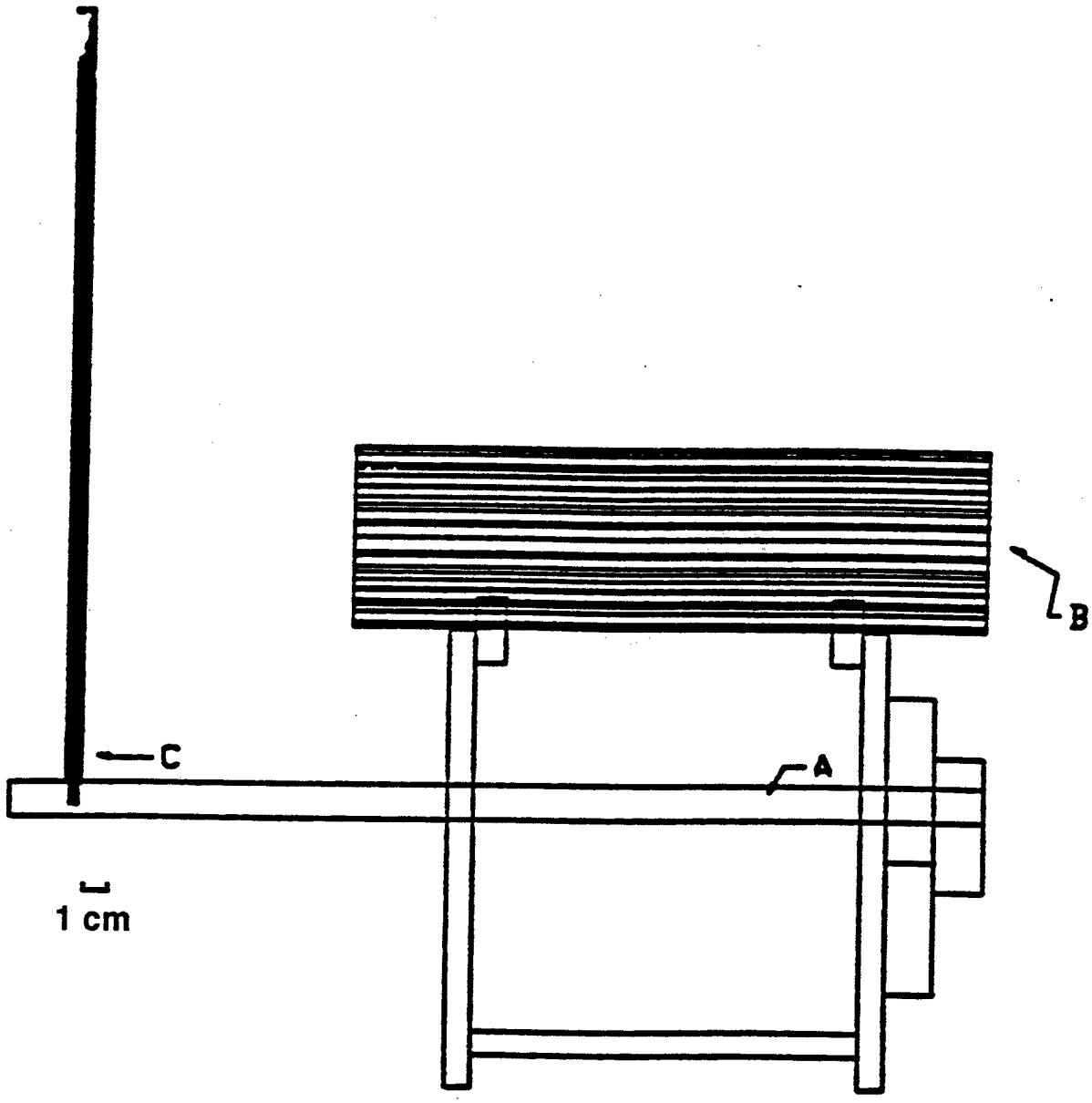
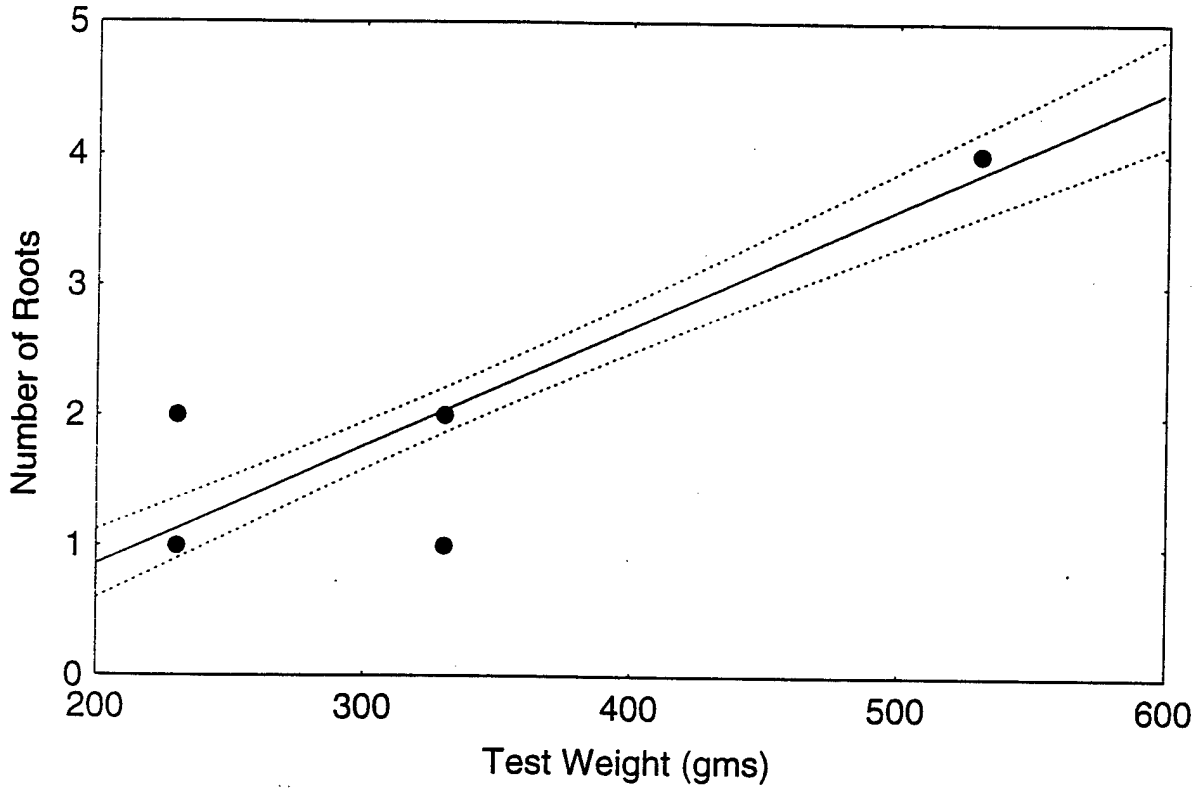


FIGURE 1



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CORRELATION OF PATIENT VARIABLES WITH GAP-43 AND P-75 IN HUMAN NEUROMAS. R.W. Beuerman*, H.S. Gilmer-Hill, R. Teal, J. Jiang, and D. Kline. LSU Eye Center, Dept. Of Neurosurgery, Neuroscience Center of Excellence, SUN Program, LSU Medical Center School of Medicine in New Orleans; New Orleans, LA 70112.

GAP-43 and p75 are proteins that promote growth cone formation and axonal elongation. The objectives of this study were to determine whether GAP-43 and low-affinity nerve growth factor receptor, p75, are elevated in traumatic human neuromas and to determine if there is a correlation between the relative amounts of these proteins and patient variables such as pain or the time between trauma and repair. Traumatic neuromas from 21 randomly selected patients were studied and the charts reviewed. Specimens were collected at the time of nerve resection and grafting, and rapidly frozen. Immunohistochemical analysis was performed on each sample, as well as on a sample of normal human nerve, using antibodies to GAP-43 and p75. In addition, Western blots were carried out. The results showed that all neuroma specimens harvested within 13 months of injury exhibited markedly elevated GAP-43 levels compared to normal nerve. Specimens harvested at 14 months or more after injury showed a precipitous drop in GAP-43 levels compared to that of normal nerve. The correlation between the amount of intra-axonal GAP-43 and post-injury time interval was significant ($p=0.0038$). High GAP-43 levels were also correlated with transection injury and pain. p75 remained elevated in our population, with no consistent pattern of variation. These preliminary data suggest that the expression of GAP-43 varies after injury, but gene expression in the dorsal root ganglion remains elevated for approximately a year and then decreases to normal or subnormal levels.

EY04074 (NIH, NEI); DAMD17-93-V-3013 (Department of the Army, DOD).

Key Words: (see instructions p. 4)

1. Axonal transport
2. Growth cone
3. Regeneration
4. Trauma

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RESPONSE OF GAP-43 AND P75 IN HUMAN NEUROMAS OVER TIME AFTER TRAUMATIC INJURY

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Running Title: GAP-43 and neuroma

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OBJECTIVE: GAP-43 and p75 are proteins that promote growth cone and neurite formation, elongation, and arborization in regenerating nerve axons. The objectives of this study were to determine whether GAP-43 and the low-affinity nerve growth factor receptor p75 are elevated in traumatic neuromas, as well as whether there is a correlation between the relative amount of GAP-43 or p75 and demographic characteristics such as time elapsed between injury and repair.

METHODS: Traumatic neuromas from 21 randomly-selected patients were studied, and the charts reviewed. Specimens were collected at the time of nerve resection and grafting. Immunohistochemical analysis was performed on each sample and normal human nerve, using antibodies to GAP-43 and p75. Western blot and computerized gel analysis were performed.

RESULTS: All neuroma specimens harvested within 13 months of injury exhibited markedly elevated GAP-43 levels compared to normal nerve. Specimens harvested at 14 months or more after injury showed a precipitous drop in GAP-43 levels, to that of normal nerve or lower. The correlation between the amount of intra-axonal GAP-43 and post-injury time interval was statistically significant, p=.0038. High GAP-43 levels were also correlated with transection injury, high postoperative sensory grade, and pain. p75 remained elevated without consistent decrease in our population.

CONCLUSIONS: This preliminary data suggests that the expression of intra-axonal GAP-43 varies over time after injury, remaining elevated for approximately the first year, then decreasing abruptly to normal or subnormal levels. These results correlate with clinical experience that peripheral nerves should be repaired relatively early, if repair is indicated.

KEY WORDS: GAP-43, Growth associated protein, Low-affinity nerve growth factor receptor, neuroma, nerve injury, p75

INTRODUCTION

Following injury to peripheral nerve, many complex biochemical and molecular events are set in motion for the purpose of repair and reinnervation. (43,44) After wallerian degeneration, axonal elongation is directed by events at both the nerve cell body and the local environment. (13,16,19) The trauma to the peripheral nerve results in the upregulation of a particular 43-kD protein, GAP-43, at the growing end of the regenerating axon, or the growth cone. The growth cone develops after neurotmesis as a specialized structure responsible for growth, pathfinding, and recognition of target tissue. GAP-43 is associated with neurite outgrowth, formation of the growth cone, and growth cone spreading. (1,2,3,16,22,23,40,53). A neuroma-in-continuity, a mixture of disorganized, regenerating, immature nerve fibers and connective tissue, represents the growth cone/neurite complex, attempting to extend through the site of injury. (10,16,25,31,35)

After neurotmesis, non-neuronal cells in the distal nerve stump, especially Schwann cells, synthesize neurotrophic factors. These changes in Schwann cell function have been described as switching from a "myelinating" mode to a "non-myelinating, growth-supporting" role. (16,19,20) Genes that code for myelin-associated proteins, such as myelin basic protein, are downregulated. Proteins normally expressed by non-myelinating axons, such as p75 low-affinity nerve growth factor receptor and GAP-43, are upregulated, along with nerve growth factor (NGF), neurotrophin 4/5 (NT-4/5), brain-derived growth factor (BDNF), glial-cell-line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF), leukemia inhibitory factor (LIF), insulin-like growth factors (IGFs), interleukin-6 (Il-6), actin, and tubulin isoforms. (16,17,19,20,22,46,56) GAP-43 and NGF receptors are expressed intra-axonally in motor and sensory neurons, and also in denervated Schwann cells after nerve lesion. (16,20,43,47,56) Following the initial increase in NGF after injury, both high- and low-affinity sensory intra-axonal NGF receptors are downregulated (8,11,20,44,56). Motoneurons, by contrast, respond to axotomy by upregulating synthesis of the low-affinity NGF receptor (p75) approximately 3-4 days after the initial injury. (4,43,44) The Schwann cell-secreted p75 NGFR increases after neurotmesis in all types of peripheral nerves, be they motor, sensory, sympathetic, or mixed. Although these changes in p75 and GAP-43 levels are highly consistent and well-documented in experimental studies (20,28,37,43,44,47,56), they have not previously been

documented in injured human nerve tissue, particularly with respect to time, injury type, and other demographic factors. Generally, the upregulation of these regeneration-associated proteins has been thought to be relatively transient. However, over the course of human peripheral nerve trauma, neuroma formation, and surgical repair, considerable time may elapse. Analysis of these proteins, which are made in the cell body of the nerve and the perineural tissue, may provide some information regarding the state of the remaining neuron over the course of these events. This study was undertaken to determine whether the levels of GAP-43 and p75 are elevated in human neuromatous tissue, and whether the upregulation of these proteins persists over many months. To our knowledge, this is the first study in the literature to examine these changes in GAP-43 and p75 over time in human neuroma tissue.

METHODS

Specimens

Samples of human neuroma tissue were obtained at the time of surgical repair. The tissue samples were immediately frozen in liquid nitrogen for later analysis. The tissues used in this study were from procedures for peripheral nerve repair carried out by DGK and RLT between February, 1995 and September, 1998. No selection criteria were used for the tissues entered into the study. Tissues and patients were assigned an arbitrary case number. A normal human sural nerve harvested for a surgical graft served as control.

Patients

Relevant data for statistical correlation and clinical analysis was obtained from patient records. Pre- and postoperative sensory grades and lesion grades were assessed using the Louisiana State University Medical Center grading scale for upper and lower extremity nerve injuries (25). Patients who suffered from painful sural nerve neuromas were assessed using the Pain Severity Scale (42,49).

Western Blot

Tissue samples were prepared by rapidly homogenizing in boiling lysis buffer (1% SDS,1.0mM sodium ortho-vanadate, 10mM Tris pH7.4) boiling for 5 min and centrifuged at 1,000 x g for 15 min to remove insoluble materials. Total proteins were determined using the BCA method (Pierce, Rockford, IL). Each sample contained 15ug total protein and 15 ul of 2 x sample buffer (250mM Tris pH6.8, 4%SDS,

10% glycerol, 0.006% bromophenol blue, 2%B-mercaptoethanol). Tissue samples were then boiled for another 5 minutes. The samples were electrophoresed using SDS-PAGE on a 7.5-10% gel. Blots were transferred to nitrocellulose membrane (Hybond-ECL, Amersham Life Science, Arlington, IL), then removed from the transfer apparatus, immediately placed into blocking buffer (5% dried milk, 0.1%BSA,10mMTris ,100mMNaCl, pH7.5, 0.1% Tween-20), and incubated 1 hour at room temperature. Antibodies were prepared (anti-GAP-43 at 1:1,000 dilution, Transduction Laboratories, Lexington, KY and anti-NGF receptor p75 at 1:1,000 dilution, Upstate Biotechnology Incorporated, Lake Placid, NY) in the appropriate blocking buffer and incubated 1 hour at room temperature. The primary antibody solution was decanted and the blot washed with TBS-T buffer (10mMTris, 100mMNacl, pH7.5, 0.1%tween-20). After washing, the blots were incubated with peroxidase-conjugated affinity purified secondary antibody (1;2,000, Amersham), diluted with 5% nonfat dried milk in wash buffer, and incubated 1 hour at room temperature. Membranes again were washed using the ECL kit and the blots exposed to X-ray film for detection. Blots were analyzed using the computer-aided densitometry C Signal Gel, SPSS, Arlington, VA). Rat brain was used for a positive control for GAP-43, and negative control membranes were treated as above except for the omission of the primary antibody (47)(Figures 1and 2).

Immunohistochemistry

The tissue specimens were fixed in 4% paraformaldehyde overnight in PBS at 4°C. After washing in buffer, the specimens were embedded in OCT compound and 12μm frozen tissue sections were collected on glass slides. The three-stage avidin-biotin complex (ABC) method was used throughout. Briefly, the tissue sections were washed and placed in a moist chamber. The sections were then blocked in 0.1M PBS containing 0.5% normal goat serum for 30 min at room temperature. Affinity-purifed primary antibody was applied in 0.1M PBS solution (anti-GAP-43 1:200 , anti-NGF-R p75 1:200) and incubated overnight at 4°C. After washing with 0.1M PBS, the sections were incubated with anti-mouse IgG secondary antibody 1:200 (Vector Laboratories, Burlingame, CA) 1-2 h at room temperature, washed again, and incubated with biotinylated horseradish proxidase complex (ABC kit, Vector) for 30 min at 37°C. After washing, the sections were stained with 3-amino-g-ethylearbazale (AEC kit,Vector) washed, mounted, and viewed under a light microscope. For control slides, the sections were treated without primary antibody.

Double-Labeling Immunofluorescence

Tissues were fixed, and sectioned as described. The tissue sections were washed with 0.1M PBS and incubated with 0.5% solution of normal goat serum in 0.1M PBS for 30 min at room temperature. Sections were incubated overnight with two different primary antibodies mixed together and diluted with 0.1M PBS, 1% BSA, 0.3% triton x-100 (polyclonal anti-NGF-R p75 I:200, Chemicon International INC, Temecula, CA, and monoclonal Anti GAP-43 1:200, Sigma, St. Louis, MO) at 4°C. Antibody binding was detected by incubating the sections with fluorescein-conjugated secondary antibody (anti-mouse IgG FITC for GAP-43 and anti-rabbit IgG Texas red for NGF-R p75, Vector) diluted with 0.1M PBS, 1%BSA and 0.3% triton x-100 for 1 hour at room temperature. After final washing with 0.1M PBS the sections were examined using a fluorescence microscope (Nikon, Inc., Melville, NY). On control sections, each primary antibody was replaced separately by 0.1M PBS. (Figures 3a and 3b)

To ensure that the results were not skewed by substrate variability over time, peripheral neurofilament was analyzed by Western blot and immunofluorescence analysis as above.

Statistical Analysis

Statistical analysis of the patient data and the density values obtained from the scanned Western blots were placed into a matrix and different hypotheses regarding the data were tested using Statistica (StatSoft, Tulsa, OK). The relationship between GAP-43, p75, and the time interval between injury and surgical correction was determined by multiple regression after ensuring linearity of variables. The relationships between several variables was explored using canonical and discriminant analysis, available in the same set of statistical programs.

RESULTS

Clinical data associated with the neuroma tissue samples is summarized in Table 1. Twenty-one patients were studied, 16 males and 5 females. Ages ranged from 5-56 years of age, with a mean age of 25.8 years. Nerve injuries consisted of 15 stretch injuries, 5 transections, and 1 contusion. Follow-up was obtained by chart review. Average length of follow-up was 14.8 months, with a range of 4-37 months. Patient #6 was lost to follow-up. Four other patients (12, 13, 14, and 15) underwent surgery relatively recently, such that no evidence of nerve regeneration would yet be expected. Two patients (10, 16) had

sural neuromas, so their lesions had no motor component. Regeneration was not assessed in these two patients. Delayed initial presentation to our center prevented early repair is some cases; consequently, the mean time interval between injury and operation for repair was 10.1 months, with a range of 0.75-21 months (Figure 1.)

GAP-43 is a gene product that is primarily produced in the soma of the neurons in the dorsal seat ganglion. Peripheral nerve injury leads to increased transcription of this gene and transport of the protein to the growing tip of the axon. Immunohistochemistry for human GAP-43 produced a positive response in all of the neuroma tissues and the staining intensity was much greater when compared to short segments of normal human nerve (remaining from the nerve repair) (Figures 1 and 2). However, relative amounts of the GAP-43 protein within the neuroma tissue sample were compared directly using Western blots (Figure 3). The densities from these blots were determined by scanning the blot to provide values for statistical analysis.

Regression analysis, Pearson-product moment, showed a significant association of the variation in the levels of GAP-43 from the scanned blots and the time between injury and surgery ($r = -0.77$; $p=0.0038$) and Figures 4 and 5. The level of GAP-43 was elevated in samples from all of the patients who underwent surgery within 13 months after their injuries. After 13 months, there appeared to be a striking decline in GAP-43 protein and, of the 4 patients who underwent surgical repair later than 13 months after their injuries, 3 exhibited lower GAP-43 densities than the normal control.

In contrast, no significant correlation was found between p75 levels and length of time between injury and repair. p75 is produced in both dorsal root ganglion cells and spinal motor neurons. There was a tendency toward an increase in p75 levels after injury. Interestingly, neuroma samples from patients operated on as long as 21 months after injury still displayed markedly elevated p75 levels when compared to the normal human nerve (Figure 6.) However, the patient who underwent nerve repair 3 weeks after injury (#4), in spite of a high GAP-43 level, showed a low level of p75. Other than the general tendency for elevated p75 levels associated with the neuroma tissue, there was no relationship between any of the patient variables and p75 level. No significant correlation was found between GAP-43 and p75 levels. However, p75 may be generated from two sources in the neuroma: the intra-axonal compartment and the non-

neuronal compartment, the surrounding Schwann cells. Since these two sources of the p75 protein were intermixed in the neuroma, the contributions of these two sources could not be determined.

There was a significant correlation between GAP-43 and injury type. GAP-43 was strongly associated with transection injury (p=.02). There was no statistical correlation between GAP-43 or p75 levels and site of injury, age, or sex. However, as seen in Figure 7, a relationship was established for this sample between GAP-43, injury type and the report of pain.

There was a trend towards higher postoperative lesion grade and elevated GAP-43 level, which did not attain statistical significance. Taking into account EMG findings of patients showing little or no post-operative clinical improvement, the trend became even stronger (Table 2). Notably, the 1 patient out of 4 undergoing surgery more than 13 months after injury who had an elevated GAP-43 level, #2, has made significant clinical recovery, from lesion grade 2 to 4. It is probable that the current length of patient follow-up (4-37 months) limits this analysis. Preoperative lesion grade was directly related to postoperative lesion grade, p=0.05. There was no correlation between pre- and postoperative sensory grades.

There was a positive correlation between pain and GAP-43 level (p=.0033), postoperative sensory grade (p=.01), p75 level (p=.0037), and injury type (transection, p=.00001). The relationship between pain and transection injury (rather than stretch) was extremely significant as well (p=.0086), possibly due to the association between both of these two variables and GAP-43 levels. There was no correlation between pain associated with the neuroma and age, sex, preoperative sensory grade, time elapsed between injury and surgery, or site of injury.

Immunofluorescence and Western blot analysis of neurofilament immunoreactivity was performed using the prepared samples, to ensure that there was no systematic variation in the amount of neural tissue in the neuroma tissue samples which could account for the differences in GAP-43 and p75 over time. When the Western blots were scanned and the densities analyzed, no correlation between substrate of p75 or GAP-43 with the Western blot values for neurofilament protein was found. Values for neurofilament protein were obtained from all of the neuroma samples.

DISCUSSION

In order for functional recovery to occur, regenerating axons must make functional junctions with their muscles (original or neurotized), and reinnervation must restore the number and size of the motor units in those muscles (14,15,16). Axonal elongation begins slowly after an initial "latent" period, but accelerates to reach a rate of 1-3 mm/day by the third day after injury. It has been suggested that the growth potential of regenerating axons is maximal at 3 weeks after injury, and therefore that the optimal time to repair a nerve is approximately 3-4 weeks post-injury. (16,19) In this study, GAP-43 was found to remain significantly elevated over normal for up to 13 months. This finding provides some fundamental basis for the clinical belief that repair should be performed within 12 months of injury. Interestingly, animal studies have shown a more rapid decline in both p75 and GAP-43 than we observed in human nerve. (6,9,19) Peak levels of GAP-43 and p75 were observed in animals at approximately 1 month following axotomy, which declined to baseline levels at 6 mos. Hassan et al. (20) documented increasing GAP-43 immunoreactivity at the endplates and in the axons of rat motor nerves 2 weeks after botulinum toxin injection, with maximal intensity 4-8 weeks after nerve injection. In our patients, very high levels of GAP-43 and p75 were also observed at 0-2 mos post-injury, but levels remained markedly elevated over baseline for up to 13 months.

Regeneration rate depends on injury type and patient age (14). If nerve repair is not performed, axons regenerate faster following an injury which preserves the continuity of the nerve sheaths and basement membrane, such as a crush or a mild stretch mechanism, than after transection (16,25). In the present study, transection injury was significantly correlated with high levels of GAP-43 ($p=0.00001$).

GAP-43 is not absolutely necessary for formation of the growth cone, but nerve cells deprived of it exhibit impaired growth cone spreading and neurite extension, and ultimately axons fail to grow in the correct pathways (2,3,16,22,23,38). Aigner and Caroni (2,3) showed that dorsal root ganglia neurons cultured in the presence of anti-GAP-43 antibody developed small, rudimentary growth cone central domains, with abnormal, fragmented peripheral lamellae. They have further shown that DRG neuron growth cones containing GAP-43 that were also exposed to NGF exhibited a striking increase in the spreading area of the phase-dense central domain, as well as accelerated growth cone and neurite elongation (3). Aigner and Arber (1) also showed a striking increase in spontaneous nerve sprouting at the neuromuscular junction and dorsal root in transgenic mice constitutively expressing GAP-43, as compared

to controls. Lesion-induced nerve sprouting and terminal arborization during reinnervation were greatly potentiated in GAP-43-overexpressing mice. GAP-43 also appears to be protective against retraction-inducing agents, such as CNS myelin-derived protein. (2,3)

Since GAP-43 has been shown in multiple animal studies (1,2,3) to contribute to neurite extension and arborization, it is tempting to speculate whether high levels of GAP-43 contribute to the formation of human post-traumatic neuromas, rather than normal regenerative nerve tissue. Disorganized growth, secondary to uncontrolled sprouting, could be the event leading to the formation of a neuroma. However, animal experiments have not shown disorganized regeneration in response to GAP-43, only increased sprouting, expansion and development of the growth cone central domains, and normal terminal arborization. (1,2) Initial neuroma formation was not observed, even when nerve tissue was exposed to abnormally high levels of GAP-43. Consequently, it is most likely that the formation of abnormal, neuromatous tissue occurs primarily due to the traumatic disruption of the guiding scaffold: the endoneurial tube. It has been well established clinically and experimentally that when the endoneurial tube is disrupted without repair, regeneration occurs poorly, ending in terminal neuroma formation (Figure5). (13,16,17,22,25,29,43,44)

The causative factors in the formation of a traumatic neuroma are unclear. Statistically, the present study showed that high levels of intra-axonal GAP-43 are associated with painful neuromas. It is known that fine, regenerating nerve fibers are hypersensitive to stimulation, i.e., tapping, over the growing end of the nerve—the Tinel's sign (26,51). Conversely, prolonged lack of a Tinel's sign along the normal path of the nerve distal to the site of injury suggests lack of axonal growth. This hypersensitivity, as well as the component of continuous pain associated with traumatic neuromas, may also be an indicator of high levels of GAP-43 within the nerve. As such, these symptoms could indicate that regeneration of the nerve is proceeding. A greater potential for growth may exist if the nerve is repaired at that time.

The role of the low-affinity nerve growth factor receptor, p75, a 75 kDa transmembrane glycoprotein (11), is still uncertain. NGF is known to promote the development and survival of sensory and sympathetic neurons, but the idea that NGF has a direct effect on injured motoneurons is controversial (5,7,8,17,30,37,42,46,50,54). NGF and GAP-43 do appear to be synergistic in their actions. High levels of NGF, NT-4/5, BDNF, and NT-3 have been shown to greatly enhance axonal outgrowth in dorsal root

ganglia and rubrospinal tract neurons. (5,27,39) Experiments studying the effects of NGF on neurite outgrowth and GAP-43 show that GAP-43 mRNA levels are increased in response to application of NGF in culture. (21,39) GAP-43-transfected pheochromocytoma 12 (PC12) cells are known to display an enhanced response to NGF, suggesting that GAP-43 may modulate the neuronal response to extrinsic trophic factors. (40) p75 may indirectly complement the aforementioned actions of NGF and GAP-43, by contributing to this ideal, regenerative environment. (19,56) p75 binds all of the neurotrophins, including NGF, with equal affinity (28,30,37). The major effect of p75 on regenerating motoneurons may not be to directly increase NGF binding but to mediate the effects of other neurotrophins, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (11,12,28,30,42,46). It is believed that p75 binds to the neurotrophins secreted by the surrounding, non-neuronal cells, transports them up the axon from the distal stump, and presents them to regenerating axons, which then bind the neurotrophins via high-affinity receptors. (16,17,42).

p75 levels have previously been noted to decline with prolonged axotomy: In rat axotomized nerve tissue maintained without innervation for 1 year, Schwann cells were noted to be highly atrophic, and not to express any p75. (19,45,48,52,55) It has been reported that p75 mRNA correlates with macrophage invasion into the site of injury, and declines within 6 months. (16,28,55) p75 levels remained elevated in most of our patients studied for over a year following injury, without correlation to injury type, age, or time elapsed from injury.

It has been shown in multiple experimental and clinical series that early repair yields better functional recovery (14,15,24,25,26,33,34,36). This finding, however, has never been correlated with specific levels of GAP-43 and p75, nor has it been previously shown that these proteins vary over time in human post-traumatic neuromas. It has remained our practice at LSU Medical Center to explore and repair major peripheral nerves within 5-8 months after stretch or contusive injury, if there is no evidence of regeneration. By this time, any element of neuropraxia will have resolved, and the surgeon will be able to accurately evaluate the injured nerve in surgery using electrophysiologic studies (24,32,41,50,51). Sharp transections should be repaired within the first 48 hours, if possible (24,25) These injuries have traditionally carried the best prognosis; however, in a civilian practice, 60-70% of nerve injuries encountered are stretch, contusive injuries rather than transections (24,25). The determination of exactly

when the patient should undergo repair is based on whether the loss is complete or incomplete, focal or lengthy, or near a potential site of entrapment. The age of the patient is also considered; younger patients tend to recover more quickly, and consequently undergo repair earlier if there is no sign of regeneration. The timing of repair is also based on an estimate of the length of time that would be necessary for the regenerating neurites to traverse the distance from injury to target organ, such that recovery can be appreciated on examination (25). When there is no voluntary contraction after a time of observation, electromyography can show voluntary recruitment or even nascent motor unit potentials weeks to months before active movement begins. (35,51) In the present study, postoperative lesion grades showed a trend towards improvement with higher GAP-43 levels, which in most cases correlated with early repair. EMG findings suggested early regeneration in several patients whose neuroma specimens exhibited elevated GAP-43 levels. It is known that nerve regeneration after repair is a 5-6 year process (25); consequently, it is not surprising that this was only a mild trend among our patient group, early in the regenerative process. With further follow-up, our patients who underwent nerve repair closer to the time of their injuries may exhibit greater overall extent of regeneration than those who underwent delayed repair. Higher intra-axonal GAP-43 levels early after axonal injury, along with elevated p75 synthesis, increased numbers of regenerating motor units, decreased target muscle atrophy, and neurotrophin support, may then be shown to contribute to improved functional recovery.

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FIGURE/TABLE LEGENDS

Figure 1: Brachial plexus neuroma in continuity, representative of the tissue sampled. Abnormal, scarred upper (U) and middle (M) trunks are seen. The C7 root was found to be avulsed. Repairs using sural nerve graft were performed from the C5 and C6 spinal nerves to the divisions of upper and middle trunks.

Figure 2: Light microscopic view of staining for GAP-43 in neuroma tissue.

Figure 3. Western blot analysis of GAP-43 from neuroma tissues.

Figure 4. Correlation showing decrease in GAP-43 level relative to time ($r = -0.77$, 95% confidence limits shown.)

Figure 5a and 5b. Immunofluorescence analysis of GAP-43 (2a) and p75 (2b) shows the distribution of each protein in neuroma tissue.

Figure 6. Correlation analysis of Western blot of p75 in patients.

Figure 7. Three-dimensional visualization of the association of GAP-43 levels, time between injury and surgery and the report of pain in this patient sample and the type of injury.

PATIENT CLINICAL DATA

#	Age	Sex	Injury Type	Painful Neuroma?	Nerve Specimen	Time from Injury to OR (mos)
1	43	male	Stretch	Yes	C7	11
2	22	male	Stretch	No	Musculocutanous	17 ½
3	17	male	Stretch	No	Peroneal	9
4	5	female	Transection	Yes	Sciatic	3 wks
5	31	male	Stretch	No	Posterior Cord	5
6	29	male	Stretch	No	Lateral Cord	13 ½
7	5	female	Stretch	No	Lateral Cord	7
8	29	female	Stretch	No	Suprascapular	6
9	29	male	Stretch	No	C5	10
10	16	female	Transection	Yes	Sural	21
11	38	male	Stretch	No	C7	8
12	17	female	Stretch	No	Peroneal	11
13	40	male	Stretch	Yes	C6	17
14	19	male	Transection	No	Median	1.5
15	56	male	Stretch	No	Peroneal	11
16	25	male	Transection	Yes	Sural	7.5
17	18	male	Stretch	No	C6	8.5
18	29	male	Contusion	Yes	Median	7
19	28	male	Stretch	No	Peroneal	10
20	32	male	Transection	Yes	Radial	13
21	11	male	Stretch	No	C6	6 ½

TABLE 1

PRE- AND POSTOPERATIVE CLINICAL EXAMINATION

Brachial Plexus (Patient #)	Lesion Grade Change	Sensory Grade Change	Postoperative EMG (+/- regeneration)
Stretch :			
1	1--------→1	0--------→0	+
2	2--------→4	4--------→4	ND
5	2---------→2	3--------→3	+
6	NO F/U	NO F/U	NO F/U
7	0---------→2	0--------→4	ND
8	0---------→3	3--------→4	+
9	0---------→0	0--------→0	+
11	0---------→0	2--------→4	ND
13	Recent	Recent	Recent
17	0---------→0	0--------→3	ND
21	0---------→1	0--------→0	ND
Transection			
14	Recent	Recent	Recent
20	3---------→5	3--------→3	ND
Contusion			
18	2---------→2	4---------→5	ND
Sciatic/Peroneal			
Stretch			
3	1---------→1	4---------→4	ND
12	1---------→1	3---------→3	ND
15	Recent	Recent	Recent
19	1---------→3	0---------→3	ND
Transection			
4	0--------→4	0---------→5	ND
10	P3--------→P0	1---------→1	ND
16	P3--------→P3	4---------→4	ND
22	Normal nerve	Normal nerve	Normal nerve

ND= Not done

TABLE 2

Figure 1

FIGURE 2

Western Blot for GAP-43

1-Human neuroma (11.5 month)
2-Human neuroma (18 month)
3-Human neuroma (9 month)
4-Human neuroma (3 weeks)
5-Human neuroma (5 month)
6-Human neuroma (14 month)
7-Human normal peripheral nerve
8-Rat brain

Figure 3

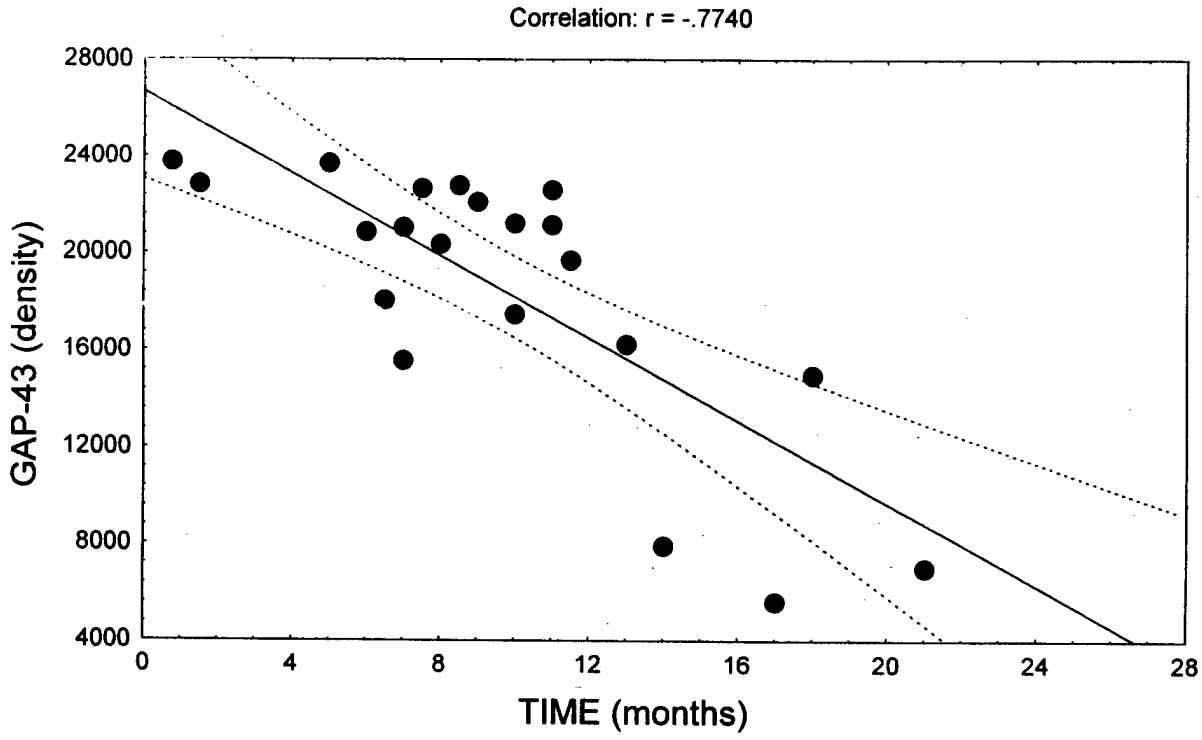
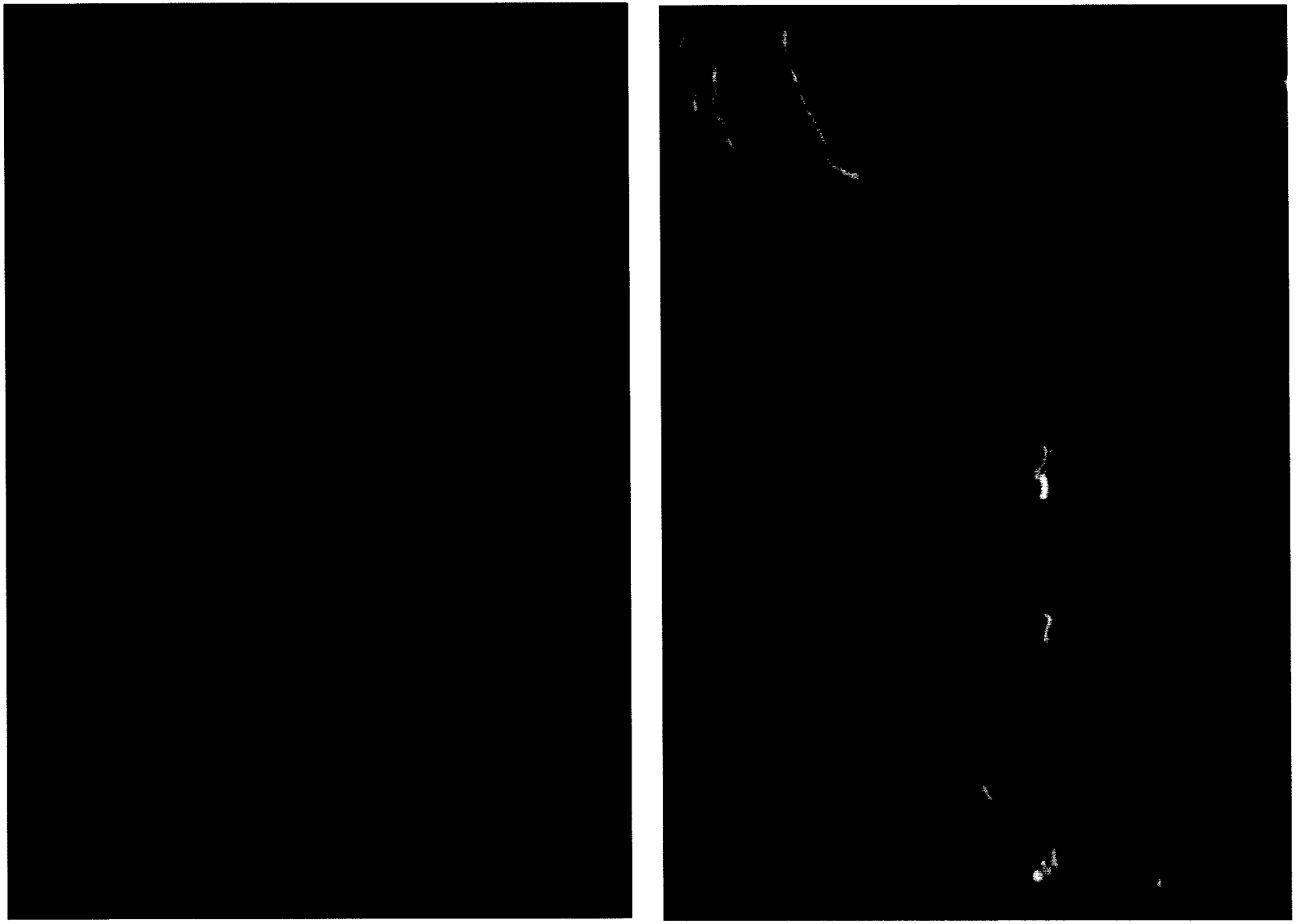

Correlation: r = -.7740
28000
24000
20000
16000
12000
8000
4000
GAP-43 (density)
0
4
8
12
16
20
24
28
TIME (months)

FIGURE 4

FIGURE 5



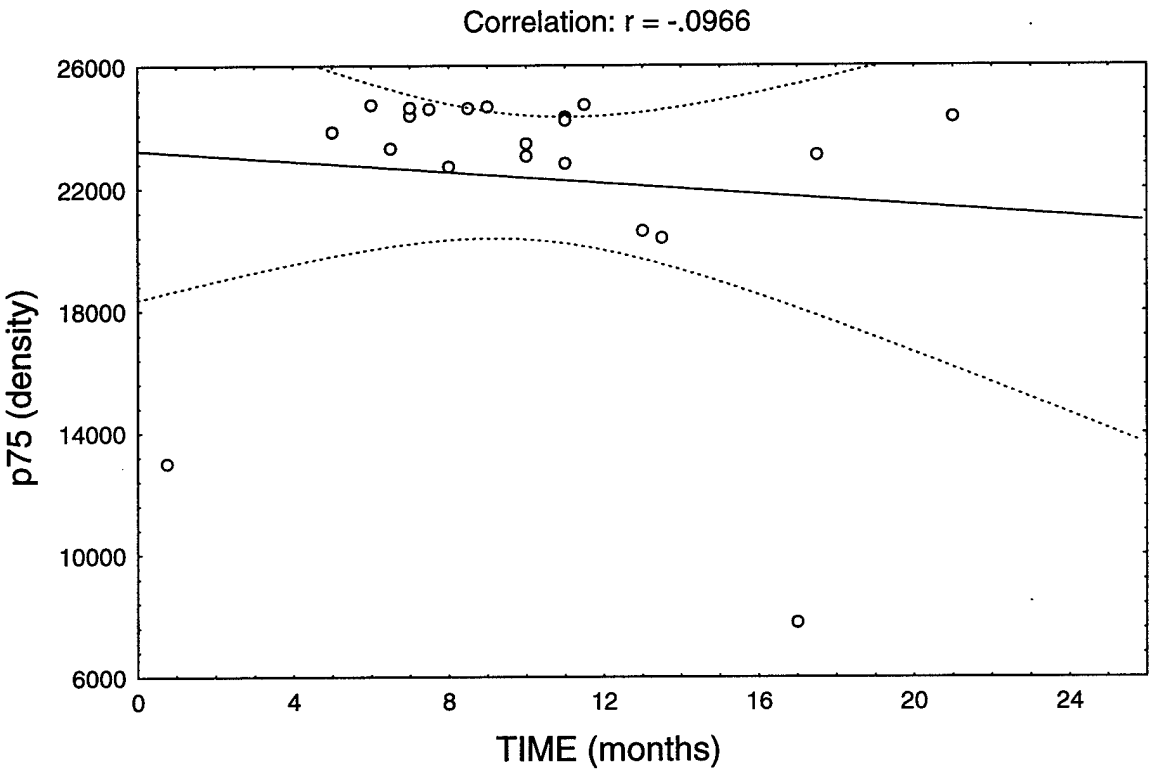
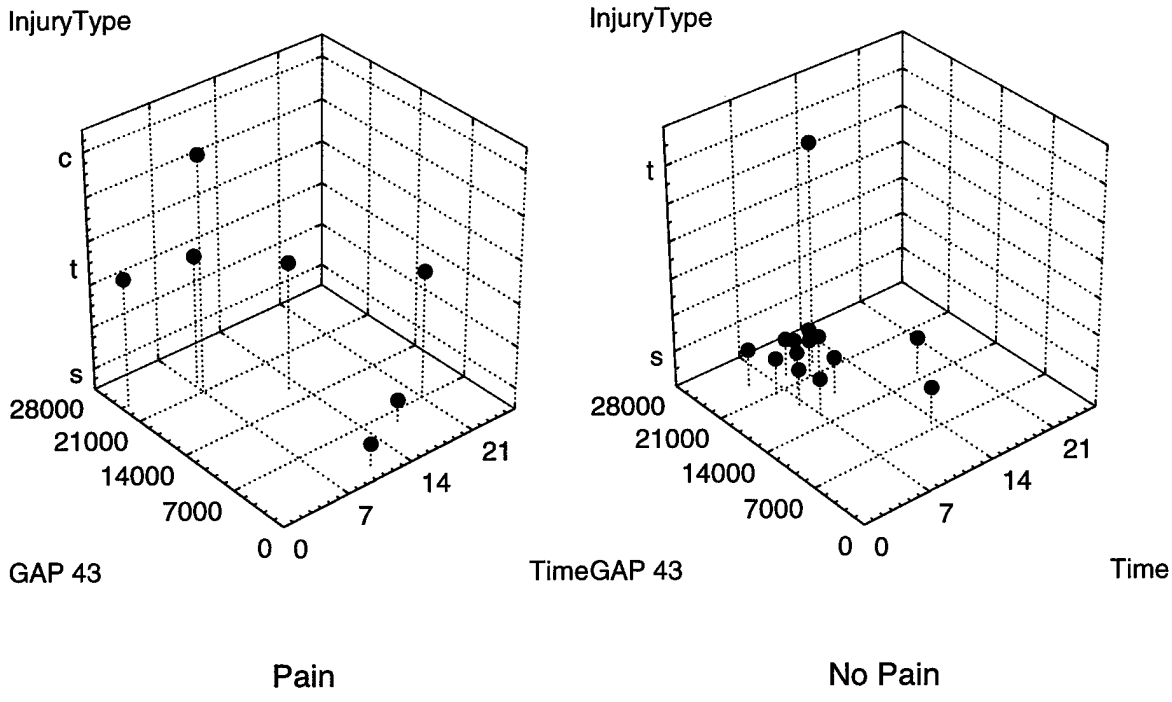

Correlation: r = -.0966
26000
22000
18000
14000
10000
6000
p75 (density)
0
4
8
12
16
20
24
TIME (months)

Figure 6



Pain

No Pain

Figure 7